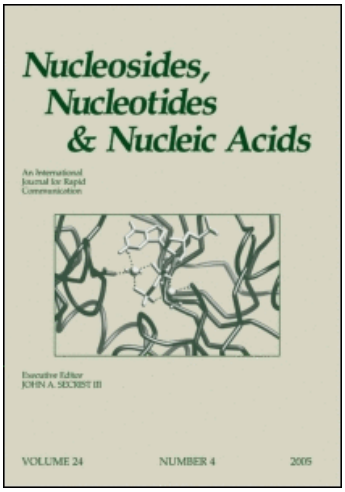

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Synthesis of High Quality Phosphorothioate Oligonucleotides as Antisense Drugs. Use of I-Linker in the Elimination of 3′-Terminal Phosphorothioate Monoesters

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ABSTRACT

Detritylation of a 5′-*O*-DMT-2′-deoxyadenosine moiety attached to solid support under acidic condition leads to depurination during oligonucleotide synthesis. Deprotection followed by reversed phase HPLC purification leads to desired oligonucleotide contaminated with significant levels of 3′-terminal phosphorothiaote (3′-TPT) monoester (n−1)-mer. However, it is demonstrated that attachment of dA nucleoside through its exocyclic amino group to solid support leads to substantial reduction of 3′-TPT formation thereby improving the quality of oligonucleotide synthesized.

Key Words: Detritylation; Depurination; Solid support; Oligomerization; 3′-terminal phosphorothioate monoester.

The revolutionary concept of antisense oligonucleotides as potential sequence-specific inhibitors of gene expression has been demonstrated with the approval of Vitravene™. First generation antisense therapeutics utilizes uniformly modified

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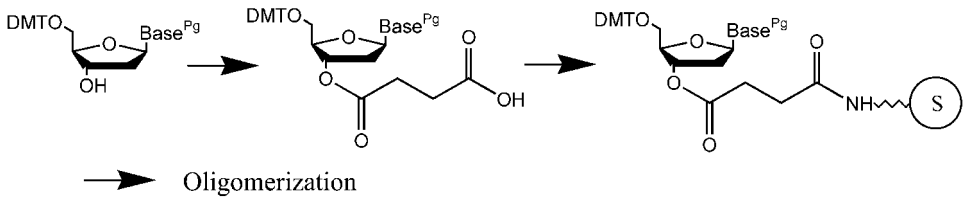

Figure 1. Standard method of oligonucleotide synthesis.

oligodeoxyribonucleotide phosphorothioates where one non-bridging oxygen atom is formally replaced by sulfur. To be successful, high quality oligonucleotides must be synthesized in large volume to meet market demand in an economical and environment-friendly way. Phosphoramidite chemistry has been widely used for the synthesis of phosphorothioate oligonucleotides due to various well-known reasons. Currently, phosphorothioate oligodeoxyribonucleotides are routinely synthesized at scales up to 300–600 mmoles in solid phase synthesizers using β-cyanoethyl protected phosphoramidites of deoxyribonucleosides with 4,4′-dimethoxytrityl (DMT) protection of 5′-hydroxyl group, benzoyl protection for exocyclic amino group of adenine (dA^{bz}) and cytosine (dC^{bz}) and isobutyryl for guanine (dG^{ibu}). At end of synthesis, the oligonucleotide is deprotected and purified by reversed-phase HPLC by taking advantage of lipophilic dimethoxytrityl group.

Capillary gel electrophoresis (CGE) analysis of ISIS 2302 active pharmaceutical ingredient (API), a 20-mer phosphorothioate oligodeoxyribonucleotide was performed to analyse the quality of the purified drug. In addition to parent and (n−1)-peaks, there was a significant amount of an earlier eluting peak. We reasoned that this peak theoretically could not be an (n−3) or (n−4)-mer. Isolation by slab gel and characterization by analytical methods revealed that the molecule to be the corresponding 19-mer with a pendent terminal phosphorothioate monoester. It is well known that removal of 5′-dimethoxytrityl group from N^6-benzoyl-2′-deoxyadenosine attached to solid support via a succinyl or related linker is accompanied by significant depurination. The depurinated oligonucleotide further undergoes elimination of sugar residue during ammonium hydroxide treatment to form 3′-terminal phosphorothioate monoester (3′-TPT) which is difficult to remove by reversed-phase chromatographic separation. The formation

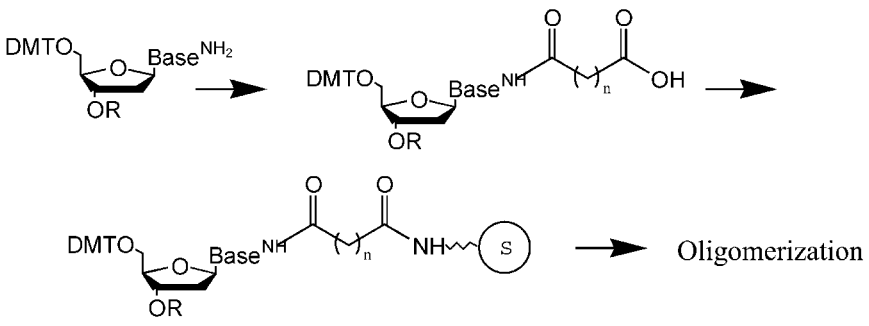

Figure 2. Oligonucleotide synthesis using I-Linker solid support.

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of this process related impurity is an inherent problem of oligodeoxyribonucleotides that start with purines at the 3′-end. Several groups have tried to minimize the level of depurination e.g., by altering exocyclic amino protecting groups. Herein we report a novel approach to eliminate the formation 3′-TPT thereby increasing the quality of antisense drugs.

Conventionally, 5′-O-dimethoxytrityl protected deoxyribonucleosides are attached to solid support via a succinyl linker attached to 3′-hydroxyl with an acyl protection on the exocyclic amino functionality (Fig. 1). We hypothesized that if we could inverse the protection/attachment chemistry we could eliminate the formation of 3′-TPT. Thus, we modified the molecule such that the linker is covalently attached to exocyclic amino group with an acetyl protection on 3′-hydroxyl group (Fig. 2; I-Linker). In this way, during acid treatment to remove DMT group, any depurination occuring will lead to loss of sugar residue with the nucleobase still attached to the solid support. This heterocyclic moiety is incapable of growing and is removed during chromatographic purifcation.

SYNTHESIS OF I-LINKER SOLID SUPPORT

Initially, we tried to synthesize standard succinyl derivative of 5′-*O*-DMT-3′-*O*-acetyl-2′-deoxyadenosine by reacting with succinic anhydride. However, no loading of nucleoside was observed as indicated by absence of orange color after acid treatment. Upon careful analysis of the molecule it appears to form a cyclic *N*-diacyl derivative thereby rendering the molecule incapable of loading on to the solid support. Similar phenomenon was observed with other shorter chain anhydrides. Longer chain derivatives did not have such problem as shown by their loading. Thus, treatment of dodecanedioyl dichloride with 1*H*-tetrazole in presence of triethylamine followed by addition of 5′-*O*-DMT-3′-*O*-acetyl-2′-deoxyadenosine afforded the desired product in ca 60% yield. Optimization of this step is currently being undertaken. Loading of this molecule to amino derivatized HL30 solid support was performed using standard conditions (loading = 90 μmole/g).

TESTING OF HYPOTHESIS

A phosphorothioate oligodeoxyribonucleotides, [(TTT-TTT-TTT-TTT-TTT-TTT-A) was chosen as an example. Six synthesis were performed on OligoPilot II DNA/RNA synthesizer. In first synthesis standard cycle for detritylation of first nucleoside (dA) attached to solid support was done (2 min) (Oligo #A). In the second synthesis, this first detritylation time was extended to 30 min keeping all other subsequent cycles similar (Oligo #B). In third synthesis, the first detritylation time alone was extended to 60 min. (Oligo #C). The fourth (Oligo #D), fifth (Oligo #E) and sixth (Oligo #F) synthesis were performed similar to first, second and third synthesis respectively except that I-linker solid support was used instead of the standard support. All oligonucleotides were purified by reversed-phase HPLC by collecting total DMT-on peak; no fractionation was done. The oligos were then analyzed by CGE and LC-MS (Fig. 3).

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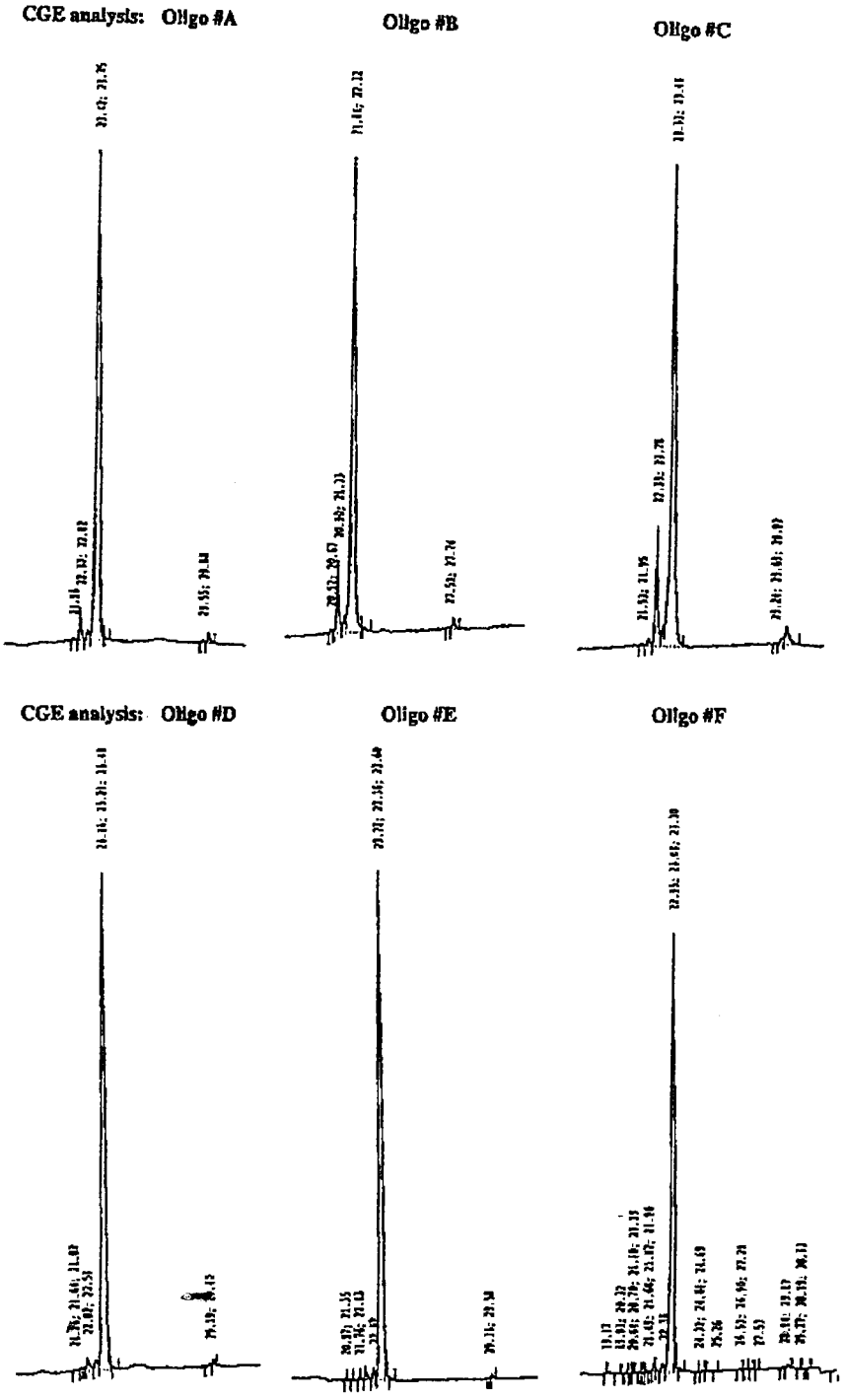

Figure 3.

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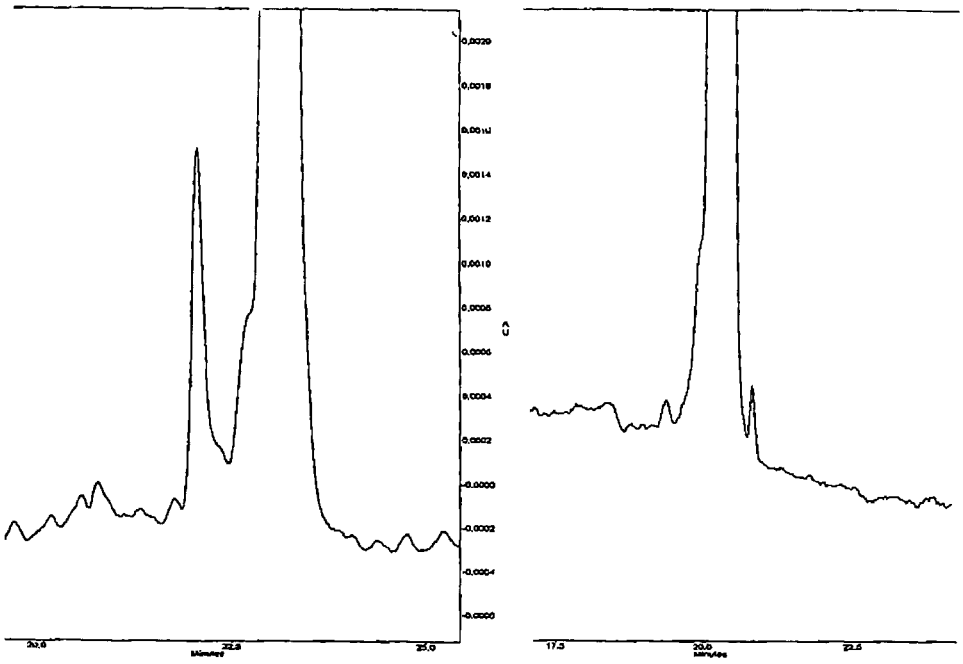

Figure 4. Synthesis of ISIS 2302: (a) Using standard support (b) Using I-linker solid support.

Next, ISIS 2302, d(GCC-CAA-GCT-GGC-ATC-CGT-CA) P=S was synthesis using the standard support and I-linker solid support. After similar purification as above, the oligonucleotides were analyzed by CGE (Fig. 4).

CONCLUSION

Based on the extensive data shown above it appears that 3′-TPT could be eliminated leading to increased quality of the oligonucleotide drug.

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